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# Micronutrient status assessment in humans: Current methods of analysis and future trends

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Summary

Micronutrients are essential to health at every life stage and their deficiencies are associated with increased incidence of various pathophysiological states and poor quality of life. Efficient methods are therefore needed to monitor micronutrient status of individuals and to improve evidenced-based recommendations for populations. This review (i) reports current approaches to assess the vitamin and mineral status in humans, (ii) summarizes current analytical advantages and shortcomings and (iii) provides practical information for both nutrition research and nutritional status diagnostics. Future analytical perspectives are discussed in relation to micronutrient profiling, analytical sensitivity, and miniaturized technologies.

**Introduction**

Vitamins and other micronutrients provide the foundations for optimal human nutrition and long-term health. Quantitative assessment of micronutrients is used to diagnose deficiencies or excess, as well as to establish dietary guidelines by regulatory agencies, e.g., the United States Food & Drug Administration (FDA). Micronutrients can be quantitatively measured in various biological matrices such as blood, urine, saliva, cells, hair, and nails. Given the long history of the effects of nutritional deficiencies and insufficiencies on health and over 100 years of research, important knowledge gaps remain on how to measure the status of several micronutrients efficiently and accurately. A lack of scientific consensus also exists on whether micronutrient functional markers could substitute for classical status markers to assess deficiencies or insufficiencies. More efforts are required to discover and validate novel functional markers that complement established status markers (i.e. indicative of specific micronutrient body stores) for the general population, specific patient groups, and ultimately individuals.

The current pandemics of non-communicable diseases such as type 2 diabetes, obesity, and cardiovascular disease associate with specific micronutrient deficiencies and/or insufficiencies, and paradoxically in some cases, overnutrition. The multifactorial characteristics of non-communicable diseases and the inherent intricacy of the absorption and activities of micronutrients from complex mixtures of food and beverages require a shift of the research paradigm from a reductionist, single-nutrient approach to studying multiple nutrients simultaneously at the system level. Nutritional management of health in future will increasingly integrate holistic micronutrient profiling with individual specific lifestyle and dietary habits. At a time when postgenomic analytics, including proteomics and metabolomics, have enabled comprehensive molecular phenotyping of humans, the field of micronutrient analysis continues to use relatively classic methodologies. A portfolio of methods is used which range from simple immunoassays to more sophisticated high-performance liquid chromatography coupled to -tandem mass spectrometry (HPLC-MS/MS) techniques[1,2]. These methods analyze (usually) a single or limited number of micronutrient entities at once. The limited throughput analysis has a direct impact on cost and required sample volumes for complete micronutrient status analysis. However, recent advances in chromatographic and mass-spectroscopy techniques offer a broad range of solutions to revisit the field of micronutrient status analysis. This review describes current markers and methodologies for the analysis of biologically important micronutrients, i.e. vitamins and minerals, in humans. A structured overview of the biological background of selected micronutrients, current status- or functional markers is provided, with the corresponding analytical methodologies, highlighting advantages and limitations. We also introduce technological perspectives related to quantitative profiling approaches, sensitivity challenges and miniaturized technologies for nutritional status analysis.

## Water-soluble vitamins

### B vitamins

The B vitamin group consists of water-soluble organic molecules that act as cofactors for many metabolic and physiologic functions[3,4].

### Thiamine (vitamin $B_1$)

Thiamine has a central role in the metabolism of carbohydrates, branched-chain amino acids and fatty acids. Thiamine occurs in the body in free and phosphorylated forms (mono-, di-, tri-phosphate and adenosine thiamine triphosphate (AThTP). Thiamine diphosphate (TDP) is the biologically active form acting as a coenzyme for, e.g.,

pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-ketoacid dehydrogenase complex and transketolase (TK). TDP is required for reactions in mitochondrial oxidative decarboxylation, pentose phosphate pathway and citric acid cycle[5]. In mammalian tissues, the concentration of thiamine triphosphate (TTP) is regulated by a specific thiamine triphosphatase[6] but knowledge gaps remain on the actual role of this functional vitamin analog. Body stores of thiamine are limited and deficiency usually occurs within 2-3 weeks of cessation of intake. Thiamine status is typically determined by measuring the thiamine dependent erythrocyte transketolase activity (ETKA), or alternatively thiamin (free or phosphorylated) concentrations. The ETKA assay provides a functional assessment of thiamin deficiencies by measuring the relative increase of erythrocyte transketolase activity in response to *in vitro* addition of TDP. Currently, this method is the most accepted for assessing thiamine deficiency although analytical variability is reported due to standardization and sample stability issues[4,7]. HPLC-based methods to quantify free thiamine, thiamine monophosphate (TMP), total thiamine (free thiamine + TMP), TDP and TTP in plasma, erythrocytes and whole blood have also been developed with the premise of providing a more sensitive and specific index of thiamine intake and status relative to ETKA[7]. HPLC analysis of TDP in whole blood or erythrocytes relies on fluorescence detection which requires pre- or post-column conversion of thiamine to thiochrome[7]. This method although sensitive requires further improvements related to inter-laboratory standardization and throughput[8].

**Riboflavin (vitamin $B_2$)**

Vitamin $B_2$ is involved in redox reactions and antioxidant functions, metabolism of other B vitamins ($B_3$, $B_6$, $B_9$) and energy production[9]. Intracellular metabolism involves phosphorylation of riboflavin to form the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which account for most of riboflavin in plasma and tissues. FAD acts as cofactor for enzymes such as methylenetetrahydrofolate reductase (MTHFR) and the erythrocyte enzyme glutathione reductase (GR), while pyridoxine phosphate oxidase (PPO), which converts dietary vitamin $B_6$ to the biologically active form pyridoxal 5´-phosphate, is dependent on FMN[9]. Riboflavin status is often assessed by a functional test, the erythrocyte glutathione reductase activity coefficient (EGRac) assay[9]. The coefficient is calculated as the ratio between flavin-dependent GR activity in washed red blood cells (RBC) before and after *in vitro* stimulation with FAD. The ratio of FAD-stimulated to unstimulated enzyme activity indicates the degree of tissue saturation with riboflavin[10]. EGRac values and dietary intake of riboflavin are highly correlated making it a well-accepted riboflavin functional marker[4]. However, the method requires fresh erythrocytes and proper inter-laboratory standardization. The EGRac method may be less reliable under certain conditions including ß-thalassemia, slow conversion of riboflavin to FMN and FAD in erythrocytes, and glucose-6-phosphate dehydrogenase deficiency[4].

Hustad *et al*. compared EGRac with measures of free riboflavin and the coenzymes FAD and FMN analyzed directly in serum/plasma or homogenized erythrocytes using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)[11]. Plasma and erythrocyte FMN and FAD, EGRac and plasma riboflavin were found to correlate, and all $B_2$ vitamers, except for plasma FAD, were deemed suitable for the assessment of vitamin $B_2$ status in population studies.

**Niacin (Vitamin $B_3$)**

Metabolism of carbohydrates and lipids depends on niacin (pyridine-3-carboxylic acid or nicotinic acid, NA, and nicotinamide). NA is metabolized to nicotinuric acid (NUA) through nicotinyl–CoA by glycine conjugation or to nicotinamide (NAM). NAM is further metabolized to N1-methyl nicotinamide (MNAM), nicotinamide-N-oxide (NAMO), and then methylated and/or oxidized to N-methyl-2-pyridone-5-carboxamide (2-Py, the major metabolite) and N-methyl-4-pyridone-5-carboxamide (4-Py). The pyridines are the end products of nicotinamide metabolism in mammals[4].

Niacin and derivatives are measured by a variety of methods including gas chromatography (GC), HPLC using UV- or fluorometric detection, and mass spectrometry (MS) (reviewed in[12]). LC-MS/MS allows for simultaneous quantification of NA, NAM, NUA and 2-Py from human plasma collected in $K_2$EDTA (ethylenediaminetetraacetic acid dipotassium salt) blood tubes[12].

Niacin and seven of its metabolites (NAM, MNAM, NAMO, 2-Py, 4-Py, NUA, and trigonelline) can also be measured in urine using supercritical fluid chromatography (SFC) coupled to MS[13]. Several studies involving small numbers of subjects have been published providing ranges of niacin-derived metabolites in the urine[4]. Most of niacin in the urine is in the form of NMN (20–35 %) and 2-Py (35–60 %) and these ranges are due to interindividual variability in metabolizing niacin metabolites and dietary intake of the parent compounds and tryptophan, a metabolic precursor[14].

**Pantothenic acid (vitamin $B_5$)**

Pantothenic acid, as indicated by its name, is widespread in nature and occurs in the food chain mainly as part of Coenzyme A (CoA) and its derivative, acetyl-CoA. The amount bound in acyl carrier protein (ACP), as opposed to free pantothenic acid itself, significantly contributes to whole body status of this cofactor. Deficiency is rare in the general population, which explains the relatively few analytical methods and studies published for assessment of pantothenic acid status compared to other vitamins. While blood plasma is used to determine body status, urinary excretion is a measure of pantothenic acid uptake[4]. Early assays use pantothenic acid dependent micro-organisms such as *Lactobacillus plantarum* for quantification[4]. Since these assays may be prone to nonspecific interference, more specific radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) tests have been developed[4].

Analysis with GC or HPLC coupled to MS detection requires prior sample treatment with pantetheinase, which is not well suited for high-throughput analysis[15]. As isotopically labeled pantothenic acid is readily available from pantolactone and [$^{13}C_3{}^{15}N$]-ß-alanine[15], and hence HPLC-MS/MS analysis has become more widespread, especially in clinical laboratories. Analysis in blood and urine has been reviewed recently in detail[16].

**Vitamin $B_6$**

Vitamin $B_6$ is a generic term that refers to six interconvertible compounds, pyridoxine, pyridoxamine (PM), and pyridoxal (PL), and their phosphorylated derivatives pyridoxine 5´-phosphate (PNP), pyridoxamine 5´-phosphate (PMP), and pyridoxal 5´-phosphate (PLP). PLP is the active form, which serves as cofactor in more than 50 enzymatic functions, including transamination, α-decarboxylation, ß- and γ-elimination reactions. PLP-dependent enzymes play a vital role in amino acid biosynthesis and degradation, the metabolism of immune-modulating compounds and neurotransmitters, as well as organic acids, glucose, sphingolipids, and fatty acids.

Concentration of vitamin $B_6$ forms measured in circulation or urine are typically used to indicate vitamin $B_6$ status, with plasma PLP being the most commonly used parameter[4]. The concentrations of plasma PL, 4-pyridoxic acid (PA), and PLP, may provide additional information on vitamin $B_6$ distribution and homeostasis[4]. For instance, plasma PL may serve as a surrogate marker of intracellular PLP[17]. PA is associated with markers of cellular immune activation but not acute-phase inflammation[18]. Erythrocyte PLP has been proposed as a more appropriate marker of vitamin $B_6$ status than plasma PLP. Erythrocyte PLP, plasma PLP, PL, PA, and urinary PA excretion are responsive to vitamin $B_6$ depletion, repletion and supplementation[17,19,20].

Since the interpretation of individual B-vitamers may be confounded by ongoing biological processes of different physiological states, ratios between closely related metabolites may provide better insight into status. For example, the PA:(PLP + PL) ratio, termed PAr, has demonstrated particular value as a marker of inflammation induced vitamin $B_6$ catabolism[21].

Functional biomarkers of vitamin $B_6$ status based on traditional methods such as erythrocyte aminotransferase activity or the tryptophan-loading test are now rarely used due to practical challenges and standardization difficulties. The development of analytical capabilities, utilizing LC- and GC-MS/MS, has created the possibility of quantifying numerous amino acids and metabolites related to PLP-dependent pathways simultaneously. The metabolites of the kynurenine pathway and substrate-product ratios such as 3-hydroxykynurenine (HK): xanthurenic acid (XA) and HK:3-hydroxyanthranilic acid (HAA) have been evaluated as markers of vitamin $B_6$ status[22]. The diminished or negligible influence of confounders such as inflammation, body mass index (BMI), and kidney function on kynurenine metabolite ratios[22] further supports the use of such metabolite ratios.

**Biotin (vitamin $B_7$)**

Dietary biotin occurs naturally as either free or protein bound forms, the latter involving a covalent binding of the vitamin to polypeptide lysine residues. The protein bound form is degraded by digestive enzymes into biocytin that is further cleaved by biotinidase into free biotin and lysine. The intracellular biotin pool consists of enzymes-bound and free fractions, to either act as cofactor or for posttranslational protein biotinylation, respectively. Several enzymes requiring biotin (e.g. acetyl CoA-, pyruvate-, methylcrotonyl- and propionyl carboxylases) play key roles in the intermediary metabolism related to fatty acid synthesis, gluconeogenesis, amino acid catabolism and the citric acid cycle.

Biotin deficiency can result from insufficient dietary intake, inborn errors of biotin metabolism, specific drug intake and pregnancy[23]. Biotin status analysis can be measured using different methods including microbiological, GC, avidin binding, colorimetric, polarographic and isotope dilution assays[4]. Applications include analysis of either plasma or urine samples. No clear consensus has been reached on the best analytical approach for biotin status assessment. The most commonly accepted analysis is of urinary biotin and 3-hydroxyisovaleric acid. The performance of several markers of biotin status has been examined in a randomized cross-over study designed to create states of deficiency, sufficiency, and supplementation in sixteen healthy men and women[24]. The authors concluded that the quantification of biotinylated methylcrotonyl-CoA and propionyl-CoA carboxylases in lymphocytes were the most reliable markers of biotin status. Alternatively, the quantification of plasma 3-hydroxyisovaleryl carnitine, a metabolite resulting from decreased methylcrotonyl-CoA activity and impaired leucine catabolic pathway, by HPLC-MS/MS has been proposed as a potential marker of biotin status although further clinical validation is needed[25].

**Folate (vitamin $B_9$)**

The B vitamins folate ($B_9$) and cobalamin ($B_{12}$) jointly participate in one-carbon metabolism and thus have close biological links[4]. Both are usually measured concurrently, since deficiency of one will interact with blood status markers of the other. Active forms of folates consist of a parent molecule, tetrahydrofolate (THF), and its one-carbon derivatives, including formyl- (CHO), methylene- ($CH_2$), methenyl- (CH=), methyl- ($CH_3$) and formimino- (NHCH) groups covalently bonded to the N5 or N10 position of the pteroyl residue. These different cofactors can be further modified within cells by the addition of between 6 and 8 glutamate residues to the parent molecule.

Most analytical procedures measure the monoglutamate form and thus a polyglutamate hydrolysis step is usually required prior to quantification. Both serum and RBC folate provide relevant and complimentary clinical and nutritional information. Serum/plasma folate is regarded as a good indicator of recent folate status and is used as a

first-line clinical indicator of folate deficiency. 5-methylTHF monoglutamate is the predominant natural folate form in serum[4]. The RBC folate content is regarded as a good indicator of longer-term tissue status and has been extensively used both in nutritional population and clinical studies. In addition, RBC folate species can vary depending on common folate-related polymorphisms such as the methylene tetrahydrofolate reductase (MTHFR) 677C→T polymorphism, and genetic variations may affect the affinity of binding proteins used in some analytical methodologies[26]. These unmeasured cofounders in RBC folate analysis are known to contribute to large inter-laboratory and inter-method differences[27].

Folates can be measured using HPLC-MS/MS, electrochemical, or fluorescence based techniques as well as with radio- and immuno-based assays and the traditional *Lactobacillus casei* growth assay[28]. This microbiological approach measures all biologically active folate species, including di- and tri-glutamates of the species, but cannot differentiate between the species. The specificity of protein binding assays to measure 5-methylTHF in RBCs is limited by variations in residual polyglutamate length, and variations in the concentration of non-methylTHF species in RBCs, as noted above. Thus, LC-MS/MS is the methodology of choice and is expected to become the gold-standard method for folate status assessment[29,30]. Use of reference standards and proficiency-testing schemes are needed for a better comparison of folate status across populations.

**Vitamin $B_{12}$**

Vitamin $B_{12}$ comprises a group of cobalt-containing corrinoids also referring to cobalamins, the latter term being used for the biologically active cofactor. The vitamin was first crystallized as cyanocobalamin, the form technically known as vitamin $B_{12}$. Two mammalian apoenzymes, methylmalonyl CoA mutase and methionine synthase, need cobalamin as cofactor, in the forms of 5'deoxyadenosyl- and methyl-cobalamin, respectively. Methionine synthase catalyzes the transfer of a methyl group from 5-methylTHF to the cofactor cobalamin intermediate and then to homocysteine. This is a key step in the one-carbon cycle which produces methionine and THF.

Vitamin $B_{12}$ is bound to transcobalamin (TC) and haptocorrin (HC) for transport in the blood, with about 20 % being attached to TC and the rest to HC. However, only TC-bound cobalamin is actively transported into tissues and is therefore suggested to be a more relevant marker of vitamin $B_{12}$ status.

Historically, vitamin $B_{12}$ was measured using microbiological assays such as the *Lactobacillus delbrueckii* method which was later adapted for high-throughput use[4]. Measurement of the total vitamin $B_{12}$ concentration in serum is the first-line clinical test for determination of vitamin $B_{12}$ deficiency. The current assays are mostly based on competitive binding of the serum vitamin to intrinsic factor, followed by radiometric or fluorescence-based

detection. A newer method estimates holotranscobalamin (holoTC) as a fraction of vitamin $B_{12}$ carried by TC in serum and therefore available for tissue uptake. This assay is currently being evaluated in many clinical laboratories, and reports to date suggest that holoTC is a more reliable marker of tissue vitamin $B_{12}$ status than serum total vitamin $B_{12}$[31].

Two metabolite markers, methylmalonic acid (MMA) and homocysteine (Hcy), are also assessed routinely. MMA is derived from methylmalonyl-CoA, a substrate of the mutase enzyme that uses cobalamin as cofactor. Low serum MMA concentrations indicate normal status, whereas in vitamin $B_{12}$ deficiency the concentration rises sharply. Similarly, an increase in the total plasma Hcy concentration is also indicative of vitamin $B_{12}$ deficiency since it reflects impaired function of the methionine synthase reaction. However, Hcy is not specific for vitamin $B_{12}$ since it is also elevated in folate or vitamin $B_6$ deficiency. Furthermore, interpretation of either biomarker can be biased by confounding factors including impaired renal function, dietary intake, and genetic variation. Although holoTC appears as the most reliable vitamin $B_{12}$ status marker, more research and clinical validation is required to reach consensus on how to assess this vitamin status in general population and patient groups. Speciation analysis of the different cobalamin forms (i.e. methyl-, hydroxyl-, cyano-, and adenosyl-cobalamin) by HPLC coupled to inductively-coupled plasma mass spectrometry (ICP-MS) could be developed as an alternative approach for vitamin $B_{12}$ status. Nevertheless, such analysis in biological fluids remains challenging due to the endogenous concentrations of the different cobalamin forms at the picomolar range.

**Vitamin C**

Vitamin C (ascorbic acid) is a strong reducing agent that when oxidized generates dehydroascorbic acid and subsequently 2,3-diketo-L-gulonic acid. To minimize the loss of vitamin C through oxidation, metabolism, or excretion, physiological systems have evolved an ascorbate recycling mechanism implying a rapid reduction of dehydroascorbic acid to ascorbate in the intracellular environment by glutathione or the selenoenzyme thioredoxin reductase.

In addition to antioxidant roles, ascorbic acid participates in a variety of enzymatic reactions, especially α-ketoglutarate dioxegenases that are involved in such diverse roles as response to hypoxia, deoxyribonucleic acid (DNA) hydroxymethylation, collagen maturation, and carnitine synthesis[32]. These enzymatic functions underlie scurvy symptomology that is the result of vitamin C deficiency. Furthermore, vitamin C plays also key roles in the homeostasis of iron metabolism[4].

Several biological compartments such as whole blood, erythrocytes, leucocytes, and plasma or serum can be used to assess vitamin C status. However, serum or plasma concentrations are the most reliable marker, as tissue concentrations are typically used to infer sufficiency in cells, tissues, and other biological fluids. Analysis of ascorbic

acid in biological samples is complicated by the high susceptibility of this compound to oxidation. Methods for stabilization of ascorbic acid after sample collection are often critical to achieve reliable quantification. Ascorbic acid is preserved at low pH (typically, concentrated meta-phosphoric or perchloric acid solutions) and reducing agents such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP), followed by immediate analysis or storage (usually -70°C or -80°C)[4,33]. The addition of reducing agents precludes the analysis of native dehydroascorbic acid concentrations, but these are not considered relevant for the assessment of whole body vitamin C status. Alternatively, small amounts of EDTA can effectively prevent oxidation of ascorbic acid. The chelating agent acts by complexing divalent minerals ions such as copper which can no longer form metal–oxygen–ascorbate complexes, which are diradical intermediates in the oxidation of vitamin C to dehydroascorbic acid. Several approaches have been developed to measure vitamin C in biological materials including spectrophotometric, fluorometric, electrochemical and liquid chromatography methods[4]. HPLC using either ion-exchange or reverse phase columns coupled to spectrophotometrical, electrochemical and ultraviolet detection, provides an efficient means to quantify vitamin C with good selectivity and sensitivity[34].

## Fat-soluble vitamins

### Vitamin A and provitamin A carotenoids

Vitamin A refers to all compounds with vitamin A activity, but only retinol and its fatty acid esters are considered relevant for determining vitamin A status in humans. Night blindness and impaired dark vision adaptation are typical clinical signs of xerophthalmia, and can be assessed as a functional marker of clinical vitamin A deficiency[35]. Xerophthalmia responds within hours to days after high-potency vitamin A treatment. Plasma retinol is probably the most analyzed biochemical indicator for determining vitamin A deficiency, defined as plasma retinol concentrations < 0.7 μmol/L[36]. However, plasma retinol is not a sensitive indicator of vitamin A status in individuals since the plasma concentration is under strict homeostatic control[36] and is depressed during infection and inflammation[35]. Pro-vitamin A carotenoids contain one unsubstituted ß-ionone ring and also contribute to retinol status. The most important pro-vitamin A carotenoid is ß-carotene, followed by ß-cryptoxanthin, α-carotene and γ-carotene. The biological activity of these compounds relative to retinol is estimated to be in the order of 50% for ß-carotene and 25% for carotenoids with only one ß-ionone end group[37].

Retinol can be measured using dried blood spots (DBS), providing the ability to collect small blood samples under field conditions with limited infrastructure[38]. Although this approach has therefore received considerable attention

as vitamin A status test in remote areas, stability issues limit the widespread practical application[38]. The retinol-binding protein (RBP), an independent measure of vitamin A concentration, has shown good sensitivity and specificity for predicting vitamin A deficiency under field conditions for 2–6 weeks after DBS collection. However, RBP is depressed during Zn deficiency and inflammation, and could also be affected by kidney disease and obesity, decreasing its utility as surrogate marker for vitamin A deficiency[35]. HPLC coupled with UV, diode array or fluorescence detection is used to measure retinol concentrations in plasma[4]. The retinol metabolites retinal and retinoic acid as well as further related oxidation products can be quantified down to the femtomolar level in serum, tissue, and chylomicrons by LC-MS/MS methods[39,40].

Carotenoids are mainly assessed from plasma using HPLC coupled to visible spectrophotometry. The distinct color and high molar absorption coefficient of these compounds make this detection competitive to MS/MS detection with regard to sensitivity and specificity with the benefit of avoiding the use of isotopically labeled standards[41].

The retinol isotope dilution technique (RID) can assess total body stores of vitamin A, detect quantitative changes in response to interventions, and can be used to determine the efficacy of pro-vitamin A food-based interventions[42]. The RID method is based on the oral administration of a small dose of tracer-labeled vitamin A followed by the determination of the tracer, either $^{2}H$ or $^{13}C$, to unlabeled vitamin A (as a ratio) in plasma using mass spectrometry. Most current methods involve extensive and time-consuming extraction/purification procedures. However, a recent, highly sensitive LC-MS/MS analytical method employed a one-phase extraction that did not require additional processing and allowed separation of labeled ß-carotene, retinol, retinyl acetate, retinyl linoleate, retinyl palmitate/retinyl oleate, and retinyl stearate within a 7-minute runtime[43].

Regardless of the technology for measuring retinol, a combination of biochemical analyses with model-based compartmental analysis allow quantification of the exchangeable total-body vitamin A in human subjects after oral administration of stable isotopes[44]. More importantly, using a single equation and a single blood sample 4-5 day after administering labeled vitamin A provides a good estimate of individual total body vitamin A stores, thus overcoming the major drawback of large inter-individual variability of tissue vitamin A concentration and status[45].

**Vitamin D**

Vitamin D encompasses vitamin $D_3$ (cholecalciferol) and vitamin $D_2$ (ergocalciferol). In the body, vitamin D undergoes successive metabolic hydroxylation into 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D in the liver and kidney, respectively. Vitamin D is essential for bone development and maintenance, and plays an important role in innate immunity. More than 50 additional metabolites have been described to date. The discovery of the 3-epimer of 25-OH-vitamin D, particularly relevant in infants, and its contribution to the vitamin D concentrations in adults illustrates

the complexity of the vitamin D metabolic network[4]. The first hydroxylation product of vitamin D, 25-OH-vitamin D is recognized as the best status marker. Since the body does not store 25-OH-vitamin D, the concentration in circulation (plasma or serum) can be used for status determination. Recently the free (not protein bound) circulating 25-OH-vitamin $D_3$ was used as measure of the bioavailable vitamin D[46]. It can be assessed either directly or can be calculated from total 25-OH-vitamin $D_3$, vitamin D binding protein and human serum albumin (HSA) concentrations. Both competitive chemiluminescence immunoassays and HPLC-MS/MS assays are used in clinical practice and the pros and cons of both technologies have been reviewed in depth[47]. The varying selectivity of the antibodies for 25-OH-vitamin $D_2$ and 25-OH-vitamin $D_3$ and potential for cross-reactivity with related metabolites such as 24,25-dihydroxy-vitamin D impact the repeatability between different immune-based assays. Thus, HPLC-MS/MS is currently regarded as the gold standard for vitamin D status assessment. HPLC-MS/MS methods enable fast separation and quantification of 25-OH-vitamin $D_2$ and 25-OH-vitamin $D_3$ and additional metabolites in a single run. Derivatization with Cookson-type reagents prior to LC-MS/MS, although adding an additional step in the sample preparation, can further increase the sensitivity, allowing the quantification of 1,25-$(OH)_2$-vitamin $D_3$ at nanomolar concentrations[48]. The Vitamin D External Quality Scheme (DEQAS) and the U.S. National Institute of Standards and Technology (NIST) developed reference standards and qualification procedures for vitamin D analysis. Results from DEQAS are published on a regular base, and reveal significant variation of assay performance.

Vitamin D is stable at room temperature on dried blood spots and was one of the first vitamins analyzed with this sampling method[49]. Derivatization followed by LC-MS/MS analysis even enables the concurrent quantification of additional metabolites such as the 3α and 3ß epimers of 25-OH-vitamin $D_3$[50]. The recent application of this methodology in the Food4Me nutritional study with nearly 1500 participants showed its suitability for application even with unsupervised sampling by the participants at home[51]. The possibility of assessing vitamin D status non-invasively from saliva was investigated[52], although more data will be needed to establish full equivalence to blood testing. Moreover, miniaturized immuno-based assays show good premises for future point of care determination of vitamin D status[53].

**Vitamin E**

Vitamin E encompasses eight naturally occurring vitamers - 4 tocopherols and 4 tocotrienols. Only α-tocopherol is routinely measured and used for status determination since this form is preferably maintained in circulation[4]. In contrast to α-tocopherol, the seven other vitamers are not recognized by the α-tocopherol transfer protein (α-TTP) in the liver. In addition to the liver, α-tocopherol is also stored to some extent in adipose tissue. The main function

of α-tocopherol is to act as reducing partner in redox reactions involving lipids. Polyunsaturated fatty acids are protected from peroxidation, especially in the cell membranes[54].

Since body stores and membrane content of vitamin E are difficult to assess, the concentration in circulation is used for status determination. α-Tocopherol is prominently associated with circulating lipid fractions, and therefore often reported relative to total lipids, LDL-cholesterol or triglycerides[4]. While total lipids are considered to be the best marker, total cholesterol is used in practice because it is easily and routinely measured.

α-Tocopherol concentrations in plasma allow quantification by HPLC with fluorescence or UV detection[4]. Using normal-phase HPLC, tocopherols and tocotrienols can also be separated. A recently introduced fast and sensitive reversed-phase HPLC method resolves the challenging separation of ß- and γ-tocopherol[55]. Separation and quantification of the eight stereoisomers of α-tocopherol is much more challenging. While the four 2R-isomers can readily be separated by chiral-phase HPLC, e.g., as their acetates using Chiralcel OD[56], an efficient analysis of the four 2S-isomers still must be developed. Current methodologies require quantitative preparation and isolation of the respective acetates, followed by conversion to their methyl ethers, and GC separation with a runtime of more than 2 hours[56].

Further research into functional markers for vitamin E status could lead to target compounds or assay systems that would have more potential for point-of-care applications. Examples include the measurement of products of lipid peroxidation such as 4-hydroxynonenal, malondialdehyde, or pentane as indicator of vitamin E status[57]. While some correlations to α-tocopherol status have been established, these compounds need further validation to be accepted as reference markers. So far, cell activity assays based on measuring hemolysis of erythrocytes under oxidative stress[4] and urinary α-carboxyethyl hydroxychroman (α-CEHC) appear as promising functional markers for α-tocopherol[58].

**Vitamin K**

Vitamin K is a name for a family of compounds including phylloquinone (vitamin $K_1$) from plant based foods and the menaquinones (different molecules which are referred to as vitamin $K_2$) from carnivorous and bacterial sources. The nomenclature uses MK-n, where n stands for the number of isoprenoid residues in the aliphatic side chain. Physiologically important forms of vitamin K are $K_1$, MK-4, MK-7, MK-8, MK-9 and MK-10[59]. The various forms have very different pharmacokinetics with half-life of 1-2 hours for MK-4 and $K_1$ and 3 days or more for MK-7 and longer chain MKs[60].

Vitamin K is reduced in the cell to form vitamin K hydroquinone and serves as a cofactor for the endoplasmic enzyme γ-glutamate carboxylase (GGCX), which converts sequence-specific glutamate residues into γ-carboxyglutamate (Gla) in a post-translational carboxylation reaction[4]. Different vitamers exhibit different cofactor activities. Vitamin K

insufficiency causes incomplete protein carboxylation and hence leads to sub-optimal Gla-protein activity which in turn results in impaired blood clotting.

Human vitamin K status may be assessed by measuring the circulating concentration of each of the relevant vitamers or by measuring the circulating concentration of uncarboxylated Gla-proteins[4]. Direct measurement of circulating K-vitamers is generally accomplished by reversed phase HPLC or ultra-performance liquid chromatography (UPLC) with fluorescence or mass spectrometric detection[61,62]. Unfortunately, many menaquinones are not available as reference compounds preventing their accurate analysis by MS. In addition, the concentration in circulation reflects recent dietary exposure rather than true status concentrations. Nevertheless, the level of response to dietary intake has been suggested to estimate vitamin K status[63]. The Vitamin K external quality assurance scheme (KEQAS) has been created to harmonize vitamin K1 measures across laboratories with reference samples. Similar to several other vitamins, the relationship between circulating and tissue vitamin K concentrations is not well established, limiting the ability to assess vitamin K body status. ELISA-based methods for measuring uncarboxylated Gla-proteins are currently the most reliable for assessing vitamin K status. These are direct and functional assays that assess tissue-specific proteins including descarboxy-prothrombin (PIVKA) for liver, uncarboxylated osteocalcin (ucOC) for bone, and circulating desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP) for the arterial vessel wall[64]. Many of these assays can be automated for processing large sample numbers.

## Minerals

Iron, copper, zinc, selenium, iodine and manganese are essential micronutrients involved in multiple biological processes including energy production, oxido-reduction reactions, signal transduction, electrolyte balance, structure of proteins and enzymatic catalysis[4]. Depending on the mineral, status measurements are performed by measuring either the total amount of the element or a related biomolecule, mainly proteins.

### Iron (Fe)

Fe is an integral component of heme, a cofactor of hemoglobin and myoglobin that occur in blood and muscle, respectively. In addition, iron-sulfur clusters (mainly 4Fe-4S and 2Fe-2S) are essential for the mitochondrial respiratory chain in the cells. Fe homeostasis is controlled by a complex molecular system that regulates uptake, storage, and mobilization[4]. Dietary Fe is transported from the small intestine into the enterocytes as $Fe^{2+}$ by a divalent metal transport protein 1 (DMT1) or as heme-iron through a receptor mediated endocytosis mediated by the heme carrier protein 1 (HCP1). Fe is then released by ferroportin (FPN1)[65] into circulation where it complexes as $Fe^{3+}$ with

the transporting protein transferrin. Fe status can be measured using different markers and matrices including blood serum, plasma and bone marrow. Since it is correlated with the total body Fe stores, the concentration of serum ferritin is a marker of choice for the diagnosis of both Fe deficiency and overload. Serum ferritin analysis can be performed by immunoturbidimetry, nephelometry, and electro-chemiluminescence immunoassay, each method differing in sensitivity and selectivity. Since ferritin is an acute phase protein, its use for Fe status analysis is affected by inflammation. Assessment of Fe status can be improved by measuring the blood soluble transferrin receptor (sTfR), an inflammation insensitive marker, by nephelometry. sTfR originates from proteolysis of the TfR on the surface of erythroblasts and quantification of sTfR reflects Fe demand for erythropoiesis. Both ferritin and sTfR can be combined in the sTfR/log ferritin ratio, i.e.. the sTfR Index, which was proposed as a better estimate of Fe status[66]. Recent evidence suggests that hepcidin-25, a peptide hormone acting as a main regulator of human Fe homeostasis, may be a reliable status marker for Fe status[67] pending proper clinical validation. Several quantitative methodologies for hepcidin have been developed in different biological fluids including immunoassay and MS[68].

**Copper (Cu)**

Cu is an essential mineral that is part of the catalytic centers of enzymes such as ceruloplasmin (Cp), hephaestin, cytochrome *c* oxidase (CCO) and Cu-Zn superoxide dismutase (SOD). Cu is involved in iron- and energy metabolism as well as in antioxidant defense systems. Cu is absorbed as $Cu^{+}$ through a specific transporter (CTR1 or SLC31A1) located in the enterocytes and circulates in serum as Cu bounded to albumin, transcuprein, and ceruloplasmin[69]. Cu status is mainly determined by its serum total concentration, ceruloplasmin or by the functional test of Cu/Zn SOD activity[4]. Each method has limitations due to lack of standardization and high inter-individual variability[70]. These markers can also be confounded by inflammation, pregnancy, lifestyle (smoking), as well as by physiological conditions linked to aging and pathological conditions (hypertension, cirrhosis, and cancer). Alternative biomarkers of Cu status have been evaluated but with limited success[4].

**Zinc (Zn)**

Zn is involved in many biochemical reactions acting as enzyme catalyst (e.g., Cu-Zn SOD) and structural conformation element (Zn finger transcription proteins)[4]. Zn is absorbed in the small intestine by a class of specific transporters (ZnT and Zip)[71]. Intracellular Zn transport is ensured by a family of cysteine-rich proteins (e.g. metallothioneins)[72]. In blood, Zn is predominantly transported via binding to serum albumin and alpha-2-macroglobulin, which accounts for only 0.1 % of the total body Zn, the vast majority of Zn being located in the skeletal muscle, bone, liver and skin cells[73]. Despite the high prevalence of Zn deficiency, the assessment of its status still lacks a well-accepted biomarker.

So far, serum or plasma total Zn concentrations are accepted as status markers, with demonstrated response to both supplementation and depletion[4]. Urinary Zn excretion is also considered a useful indicator of Zn status due to its collinear response to either Zn supplementation or depletion[74]. Total Zn concentration can be quantified using atomic absorption spectrometry (AAS), inductively-coupled plasma optical emission spectroscopy (ICP-OES) and ICP-MS. Surrogate indicators of Zn status including number of platelets, polymorpho- and mononuclear cells, as well as erythrocyte Zn content and plasma alkaline phosphatase activity have also been considered but with limited clinical acceptance[4]. Alternatively, a new series of markers such as erythrocyte metallothioneins, plasma extracellular superoxide dismutase, expression of Zn transporter *ZIP1* mRNA in lymphocytes and Zn-regulated low-molecular-weight humoral factor are being developed.

**Selenium (Se)**

Se is centrally involved in the intra- and extracellular redox-regulation and participates in antioxidant defense systems through glutathione peroxidase- (GPx) catalyzed reduction of hydrogen peroxides[4], where selenocysteine forms part of the enzyme catalytic site. In the enterocytes, dietary Se absorption occurs either via selenite ($SeO_3^{2-}$) or selenate ($SeO_4^{2-}$) salts or as organic compounds such as Se-Cysteine and Se-Methionine using multifunctional anion exchanger ($Na^+$-sulfate transporters)[75] and specific amino acids transporters such as the $b^{0,+}$rBAT system, respectively[76]. Once absorbed, Se is enzymatically converted into selenide ($H_2Se$), the precursor for selenoprotein biosynthesis. In the liver, Se is then incorporated into selenoprotein P (SePP) that is released in the circulation as the major circulating Se-containing molecule. Se status is determined using the serum concentration of total Se (mainly indicating short-term status) and SePP[77]. Serum Se concentration is affected by lifestyle (smoking, alcohol intake) and inflammation. Analysis of total Se can be performed using atomic spectroscopic techniques such as AAS and ICP-MS or total X-ray reflection spectroscopy whereas ELISA assays are available for SePP. The erythrocyte Se concentration has been proposed as an alternative Se status biomarker that would be less confounded by acute-phase response[78] and more indicative of mid- to long-term Se status. Long-term Se status can also be assessed using measurement of GPx activity in erythrocytes[79,80].

**Iodine (I)**

Iodine is key to the function of the thyroid gland where it is involved in the synthesis of the hormones thyroxine (T4) and triiodothyronine (T3) which contain four and three I atoms, respectively. Thyroxine plays a pivotal regulatory role for brain development and the basal metabolic rate and also promotes bone growth by interacting with the growth hormone (GH)[81]. Dietary I is readily absorbed by the enterocytes as iodide ($I^-$) by a sodium-iodide symporter

(NIS)[82]. The level of expression of NIS is modulated by the circulating concentration of iodide and by thyroid-stimulating hormone (TSH)[83]. I status is determined by measuring concentrations of I in urine or concentration of TSH and thyroglobulin (Tg) in serum[4]. Evaluation of the goiter rate is also used to assess I deficiency. Serum concentration of TSH is an indicator of I status particularly in infancy. However, I intake and serum TSH concentration exhibits a U-shaped relationship and both too low and too high nutritional I intake result in elevated serum TSH concentrations[84]. Serum quantification of Tg, the precursor of T3 and T4 hormones, is more responsive to dietary iodine and can be seen as a mid-term biomarker of I status[85]. Both serum TSH and Tg concentrations can be measured by immunoassays. Urinary iodine (UI) concentration is a valuable indicator of short-term I status. Indeed, more than 90% of dietary iodine is excreted in urine and urinary I concentration changes rapidly in response to dietary I intake. Total I in 24 h urine is considered the most reliable status marker although spot urine samples are also used in epidemiological studies with normalization to creatinine excretion[4]. Urinary I is measured by a kinetic-colorimetric assay based on the Sandell-Kolthoff reaction or by ICP-MS[4]. Long-term I deficiency can be inferred by the clinical examination of a goiter by palpation or by ultrasonography[86]. However, these methods lack sensitivity, specificity and can be confounded by several I-independent pathophysiologies.

**Manganese (Mn)**

Manganese is an essential trace element for humans. Mn is cofactor for Mn-dependent SOD2 (mitochondrial form), pyruvate carboxylase and arginase and is involved in neurological-, immune-, antioxidant and energy metabolism[87]. Mn homeostasis is tightly controlled at the levels of intestinal absorption, tissue uptake and excretion to prevent deficiency and accumulation, the latter potentially causing cellular oxidative damage[87]. Mn and Fe metabolism are closely intertwined since both metals share uptake and transport pathways. Alike $Fe^{2+}$, $Mn^{2+}$ enters the small intestine enterocytes mainly through DMT1. $Mn^{2+}$ is exported from the cells through FPN1 followed by an oxidation into $Mn^{3+}$ mediated by ceruloplasmin. In circulation, $Mn^{3+}$ is mainly bound to proteins such as transferrin and HSA and transported to tissues where uptake is mediated by several transport mechanisms, including DMT1 and the transferrin receptor[87]. Mn nutritional status is mainly assessed by its elemental quantitation in blood serum, urine or hair samples using atomic spectroscopy or elemental MS techniques. However, several studies demonstrate partial response of serum concentrations to Mn supplementation. While urinary Mn excretion is indicative of depletion, Mn determination in hair has limited value for status assessment[4]. The analysis of SOD2 concentration in peripheral blood mononuclear cells using immunoassay is foreseen to provide a Mn functional marker although lacking clinical validation[4].

**Current trends and future perspectives**

Vitamins and mineral status analyses are fundamental for nutritional status assessment. A large variety of methodologies for vitamin and mineral measurement have been developed over decades, targeting either single molecules or a reduced set of analytes. The development of quantitative profiling methods for the analysis of multiple vitamins remains a challenging task. Indeed, the intrinsic physical properties of vitamins (i.e. water- or fat-soluble molecules) imply the need for different sample preparation and analysis protocols. Furthermore, several vitamins are prone to rapid degradation due to oxygen, light and temperature. High heterogeneity exists in terms of molecular structures and concentration range (from μM to pM range) within each group of vitamins (e.g. B group vitamins). These analytical difficulties have contributed to a highly fragmented landscape of available methodologies that often lack validation and inter-laboratory standardization. In addition, the high number of micronutrient-specific methods has hampered the possibility to investigate nutrient-nutrient interactions and the influence of genetic, environment (including diet), and disease factors. Analytical sensitivity, cost and sample volume requirements of the various assays are further limitations. However, recent technological improvements of chromatography and mass spectrometry open new possibilities to perform high throughput micronutrient quantitative profiling in biological matrices[88]. Recently, a method was developed and validated for the quantification of a set of 14 liposoluble vitamins and carotenoids in a single run of 8 min from 200 μL of human blood plasma using a combination of supported liquid extraction and ultrahigh-performance supercritical fluid chromatography coupled to tandem mass spectrometry[89]. Another work reports the analysis of twenty-one water soluble vitamins by LC-MS/MS from 200 μL of plasma in less than 15 minutes[90]. Related to mineral status analysis, the recent introduction of inductively-coupled plasma coupled to tandem mass spectrometry (ICP-MS/MS) has enabled the simultaneousquantification of 29 elements from 150 μL of human blood serum with high accuracy and precision[91]. Hence, profiling of multiple vitamin forms and minerals is now possible with significantly improved analytical throughputs. Such analytical progress will continue to enhance the field of micronutrient analysis with novel profiling-derived biomarker patterns that can capture absorbed and biologically active forms and their downstream metabolites with the lowest possible number of analytical methods. Continuous developments of chromatography and mass spectrometry techniques will be key to ensure quantitation of structurally similar molecules and efficient resolution of isobaric interferences[47,92]. High resolution MS techniques will add full scan screening capabilities to the already popular isotopic dilution MS/MS solutions in the clinical assessment of micronutrient status and functions[47]. Furthermore, new methods able to differentiate speciation forms as well as isotopic signatures[93,94] of minerals could be developed to deliver complementary information on the metabolic fate of elements in biological systems. Technological developments are also expected on miniaturization of both pre-analytics (e.g. sample collection and transport) and analytics of micronutrients. DBS solutions[95] and volumetric absorptive microsampling (VAMS)[96] were developed to provide easier and faster sample processing relatively to

conventional venipuncture procedures. Technical challenges with these microsampling methods include the variability of the hematocrit content, and specifically to DBS, the accurate determination of the analyzed volume. These micro-sampling solutions are convenient in terms of cost and ease of shipment to central analytical laboratories. Usually, around 10 μL whole blood is collected, thereby challenging the sensitivity limits of analytical techniques such as MS. If one considers the recently developed micronutrient quantitative profiling methods[89,91], transfer to DBS or VAMS will result in the loss of almost half of quantifiable micronutrients with the current sensitivity limits. While more efficient sampling systems such as nano-infusion techniques are being developed, MS sensitivity will still have to increase by 100% to warrant the measure of the same analytical panels as in plasma or serum samples. However, should such method transfer to DBS or VAMS be developed, it will be essential to properly assess micronutrient stability on these sample collection devices since several vitamins are prone to rapid degradation due to light exposure and temperature.

As an alternative to central laboratory-based testing, the concept of point of need analysis holds promise to benefit micronutrient analysis with integrated, miniaturized, reliable and cost-effective measurement devices to enable personalized nutrition[97]. Of the current routine methods, immuno-based assays can be miniaturized in a compelling way and are fast enough to cope with these needs. Nano-array approaches of nano-Molecular Imprinted Polymers and aptamers that recognize and assess multiple micronutrients are also being developed[98]. Other technologies, most prominently using sensor enhanced electrochemistry, could assess many biologically active molecules in real time[99]. Moreover, it will be interesting to evaluate alternative body specimens such as saliva, sweat, hairs and breath for the minimally invasive analysis of several micronutrient status or functional markers. However, the use of these biological matrices will have to be scientifically and clinically proven of relevance in micronutrient status analysis. While promising, the general use of these new technologies would require proper methodology validation including the establishment of the equivalence of analytical performance with traditional blood assays in large population trials. Carotenoid status determination, thanks to the unique spectroscopic properties of these molecules, provides an interesting example of non-invasive analysis. Total carotenoids can indeed be assessed using RAMAN spectroscopy[100], and macular pigment optical density (MOPD) determination is now available to assess lutein contents in the retina[101].

Methodologies for micronutrient assessment are thus constantly revisited and improved towards more comprehensive profiling approaches. However, several technological and clinical challenges remain ahead for the implementation of fully validated solutions for nutrition and health management.

- achieve high levels of sensitivity to enable measurement of nutritional status in different biological compartments (blood plasma/serum, cells, and urine) and with miniaturized sample collection devices;
- quantify nutrients and micronutrients and their metabolic products to generate next generation of status and functional biomarkers;
- capture a broad range of molecules that reflect the inherent complexity of the nutritional exposome, nutrient-nutrient interactions and their effect on human health using a minimal set of analytical approaches;
- develop appropriate reference materials for quality control and proceed with a thorough validation of profiling methods to decrease inter-assay and -laboratory variabilities to ensure harmonization and data comparability;
- enable analysis of micronutrient status at point-of-need for real-time nutritional recommendations.

Should these technical challenges be efficiently overcome, future micronutrient analytics (i.e. micronutrient profiling and point-of-need devices) are foreseen to integrate with people's specific lifestyle and diet components into a holistic health management approach.

595 **Authors' contributions:**

596 UH, SR coordinated the review and wrote major parts of the paper

597 SB, BF, GL, JKa, AD, WS, DR, AJM, AMM, CV, HM, JKö, TK, AMcC contributed to the paper

598 The review is the outcome of a workshop held by DSM Nutritional Products; JKa, PW and ME

599 developed the concept of the review of current status and future steps to be taken

600 Conflict of interest:

601 UH, AD, PW and ME are employees of DSM Nutritional Products.

602 SR and TK are employees of Nestlé Institute of Health Sciences SA.

603 DR was an employee of Metanomics Health GmbH and is currently employed by BASF SE.

604 JKa was an employee of Nestle Institute of Health Sciences SA and currently is employed by

605 Vydiant Inc.

606

**Table 1:** Markers and methods for status determination of water-soluble vitamins; for details cf. the respective section in the text.

| | **Vitamin $B_1$** | **Vitamin $B_2$** | **Vitamin $B_3$ (Niacin)** | **Vitamin $B_5$ (Pantothenic acid)** | **Vitamin $B_6$** | **Vitamin $B_7$ (Biotin)** | **Vitamin $B_9$ (Folate)** | **Vitamin $B_{12}$** | **Vitamin C** |
|---|---|---|---|---|---|---|---|---|---|
| **Most commonly used marker** | Erythrocyte transketolase, erythrocyte thiamine diphosphate | Erythrocyte glutathione reductase | Plasma niacin metabolites, but not reliable | Pantothenic acid after liberation of bound forms | Pyridoxal-5"-phosphate | Urinary biotin | Serum folate (short-term status) and red cell folate (long-term status) | Serum total cobalamin gradually being replaced by holoTC Confirmatory status marker methyl malonic acid | Serum ascorbate |
| **State of the art methodology** | Erythrocyte transketolase activity coefficient (ETKac) assay or HPLC analysis of whole blood or erythrocytes | EGRac assay or LC-MS/MS analysis of riboflavin and FMN | LC-MS/MS | LC-MS/MS | HPLC or LC-MS/MS | LC-MS/MS or microbiology | LC-MS/MS and newer microbiological methods. | GC-MS for MMA; binding assays linked to fluorescence detection systems for $B_{12}$ and holoTC. | HPLC |
| **Matrix** [1] | Washed red blood cells | Washed red blood cells | Plasma, Urine | Whole blood | Plasma or serum | Urine | Serum and whole blood lysed into 1% ascorbic acid | Serum or plasma | Plasma or serum |

[1] All matrices are derived from venous blood, typically 100 µL or less are required for analysis; except for vitamin C no fasting is required prior to sampling.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Indicative concentration (matrix and marker)[2]** | 132-284 nmol/L (erythrocyte thiamine diphosphate) | 1.00-1.10 (erythrocyte EGRac coefficient) | | 1.57-2.66 µmol/L (whole blood pantothenic acid) | 30.4±14.7 nmol/L (plasma pyridoxal-5"-phosphate) | 6-50 µg / 24-hr (urinary biotine) | 13.4-44.5 nmol/L (serum folate) | 238±102 pmol/L (plasma vit. $B_{12}$) | 63.0±19.9 µmol/L (plasma ascorbic acid) |
| **Gaps / issues** | Lack of validated status cut-offs. Assay standardization against true biological function; inter-laboratory standardization required | Lack of validated status cut-offs. Assay standardization against true biological function; inter-laboratory standardization required | Validated plasma markers of niacin status | Simple sample preparation methodology | Inter-laboratory standardization required | | Analysis of red cell folates by LC-MS/MS. Issues with deconjugation of folates to mono-glutamate forms. Affinity of binding protein assays to different folate derivatives. | Factors influencing MMA that are not related to $B_{12}$ status | Inter-laboratory standardization required; results influenced by stabilization at time of collection |
| **Outlook: promising techniques or developments** | In the field alternative; point-of-care analysis; LC-MS/MS analysis of free and phosphorylated thiamine forms | In the field alternative; point-of-care analysis | In the field alternative; point-of-care analysis | | Dried blood spotting; point-of-care analysis; validation of metabolite ratios | | LC-MS/MS. Analysis of Guthrie cards is in progress but validation against serum/red cell folate is required. | HoloTC assay | In the field alternative; point-of-care analysis |

[2] Concentrations for initial guidance, not meant as reference range4.

**Table 2:** Markers and methods for status determination of fat-soluble vitamins; for details cf. the respective section in the text.

| | **Vitamin A** | **Pro-vitamin A carotenoids** | **Vitamin D** | **Vitamin E** | **Vitamin K** |
|---|---|---|---|---|---|
| **Best marker** | Total body store of vitamin A using stable isotopes | Carotenoids | 25-hydroxy vitamin D (D2+D3) | α-tocopherol ratio to total blood lipids | Uncarboxylated Gla-proteins.<br>For liver: PIVKA<br>For bone: uc-osteocalcin<br>Other tissues: dp-ucMGP |
| **State of the art methodology** | LC-MS/MS using 13C stable isotopes | HPLC-Vis | LC-MS/MS immuno-based assays | LC-UV | Immuno-based assays |
| **Matrix**[3] | Plasma or serum | Plasma or serum | Plasma or serum | Plasma or serum | For PIVKA: citrated plasma<br>For uc -osteocalcin: serum<br>dp-ucMGP: EDTA plasma |
| **Indicative concentration (matrix and marker)**[4] | 1.8±0.1 µmol/L (plasma retinol) | 378 nmol/L (serum ß-carotene) | 63.6±32.7 nmol/L (serum 25-hydroxy vitamin D) | 1.85±0.3 µg/mg (plasma α-tocopherol/ total plasma lipids) | 1.58 µg/L (plasma PIVKA), 3.3±0.4 µg/L (plasma uc-osteocalcin) |

[3] All matrices are derived from venous blood, typically 100 µL or less are required for analysis; for vitamins A, E and K and ß-carotene fasting is preferred prior to sampling.

[4] Concentrations for initial guidance, not meant as reference range4.

| | | | | | |
|---|---|---|---|---|---|
| **Gaps / issues** | Non-invasive alternatives via blood spots, isotope test dose not always at physiological concentration | Quantification of cis isomers; bioactivity relative to retinol | Standardization / variation between assays; non-invasive alternative; point-of-care analysis | Requires analysis of lipids or cholesterol for referencing | Food composition tables, notably for menaquinones<br>Stable isotope absorption studies for menaquinones<br>dietary reference values (DRV) and recommended daily allowance (RDA) for extrahepatic functions of vitamin K |
| **Outlook: promising techniques or developments** | Total body store determination through stable isotope measurements combined with modeling; application of field-friendly collection methods such as blood spots. | Spectroscopic skin tests<br>Raman spectroscopy | Dried blood spotting | | dp-ucMGP available on auto-analyzer as from 2014 (iSYS from IDS) |

**Table 3:** Markers and methods for status determination of minerals; for details cf. the respective section in the text.

| | **Fe** | **Cu** | **Zn** | **Se** | **I** | **Mn** |
|---|---|---|---|---|---|---|
| **Best marker** | Serum ferritin | Ceruloplasmin, serum Cu | Serum/plasma Zn | Plasma Se Selenoprotein P | UI | Serum Mn |
| **State of the art methodology** | ECLIA | Nephelometry, turbidimetry | AAS | AAS, ICP-MS Immunoassay | Photometric | ICP-MS |
| **Matrix** | Serum | Serum | Serum, plasma | Serum, plasma | Spot urine; 24 h urine | Serum |
| **Indicative concentration (matrix and marker)**[5] | 20.4±2.2 ng/mL (serum ferritin) | 453±16 μg/mL (plasma ceruloplasmin) | 1.17±0.42 μg/mL (serum Zn) | 50-110 ng/mL (serum Se) | 250 μg iodine / g creatine (urinary I) | 0.57±0.13 ng/mL (serum Mn) |
| **Gaps / issues** | Susceptible to inflammation / cancer | Susceptible to inflammation and other factors | Lack of specific molecular biomarker | Short- vs. long-term status, bioavailability | Previous intake of iodine-containing food | partial response of serum concentration to Mn supplementation |
| **Outlook: promising techniques or developments** | Validation of new biomarkers (e.g., hepcidin-25) | | | | Serum thyroglobulin | Clinical validation of functional markers (e.g. SOD2) |

[5] Concentrations for initial guidance, not meant as reference range4.

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Abbreviations

2-Pyr *N*-Methyl-2-pyridone-5-carboxamide

4-Py *N*-Methyl-4-pyridone-5-carboxamide

ACP Acyl carrier protein

AThTP Adenosine thiamin triphosphate

BMI Body mass index

CCO Cytochrome *c* oxidase

CoA Coenzyme A

Cp Ceruloplasmin

CSS Chaperone for superoxide dismutase

DBS Dried blood spot

DEQAS Vitamin D External Quality Scheme

DMT1 Divalent metal transport protein 1

DNA Desoxyribonucleic acid

dp-ucMGP Desphospho-uncarboxylated matrix Gla-protein

DRV Dietary reference values

DTT Dithiothreitol

EGRac Erythrocyte glutathione reductase activity coefficient

ELISA Enzyme linked immuno sorbent assay

ETKA Erythrocyte transketolase activity

ETKac Erythrocyte transketolase activity coefficient

FAD Flavin adenine dinucleotide

FDA Food & Drug Administration

FMN Flavin mononucleotide

FPN1 Ferroportin 1

GC Gas chromatography

GGCX γ-Glutamate carboxylase

GH Growth hormone

Gla γ-Carboxyglutamate

GPx Glutathione peroxidase

GR Glutathione reductase

HAA 3-hydroxyanthranilic acid

HC Haptocorrin

HCP1 Heme carrier protein 1

Hcy Homocysteine

HK 3-Hydroxykynurenine

holoTC Holotranscobalamin

HPLC High performance liquid chromatography

HPLC-MS/MS High performance liquid chromatography tandem mass spectrometry

HSA Human serum albumin

ICP-MS Inductively-coupled plasma mass spectrometry

ICP-MS/MS Inductively-coupled plasma coupled to tandem mass spectrometry

ICP-OES Inductively-coupled plasma optical emission spectroscopy$K_2$EDTA Ethylenediaminetetraacetic acid potassium salt

LC-MS/MS Liquid chromatography coupled to tandem mass spectrometry

MMA Methylmalonic acid

MNAM N1-Methyl nicotinamide

MOPD Macular pigment optical density

MS Mass spectrometry

MTHFR Methylenetetrahydrofolate reductase

NA Nicotinic acid

NAM Nicotinamide

NAMO. Nicotinamide-N-oxide

NIS Sodium-iodide symporter

NIST National Institute of Standards and Technology

NUA Nicotinuric acid

PA 4-Pyridoxic acid

PAr PA:(PLP + PL) ratio

PIVKA Descarboxy-prothrombin

PL Pyridoxal

PLP Pyridoxal 5´-phosphate

PM Pyridoxamine

PMP Pyridoxamine 5´-phosphate

PNP Pyridoxine 5´-phosphate

PPO Pyridoxine phosphate oxidase

RBC Red blood cell

RDA Recommended daily allowancesRIA Radioimmunoassy

RID Retinol isotope dilution technique

SePP Selenoprotein P

SFC Supercritical fluid chromatography

SOD Superoxide dismutase

sTfR Soluble transferrin receptor

T3 Thyroid hormone triiodothyronine

T4 Thyroid hormone thyroxine

TC Transcobalamin

TCEP Tris(2-carboxyethyl)phosphine

TDP Thiamine diphosphate

Tg Thyroglobulin

THF Tetrahydrofolate

TK Transketolase

TMP Thiamine monophosphate

TSH Thyroid-stimulating hormone

TTP Thiamine triphosphate

ucOC Uncarboxylated osteocalcin

UI Urinary iodine

UPLC Ultra-performance liquid chromatography

VAMS Volumetric absorptive microsampling

XA Xanthurenic acid

α-CEHC α-Carboxyethyl hydroxychroman